

The University of Chicago

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RATE OF CLEAVAGE AND EARLY
DEVELOPMENT OF FROG EGGS
(RANA PIPIENS)

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY
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SOME GROUP EFFECTS ON THE RATE OF CLEAVAGE AND EARLY DEVELOPMENT OF FROG EGGS (*RANA PIPIENS*)¹

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AN OPTIMAL density has been demonstrated for speeding cleavage and early development of sea-urchin eggs. Five species have been included in the work of the following investigators: Frank and Kurepina (1930), Maxia (1933), Allee and Evans (1937*a* and *b*), and Sugiyama (1938). The question arose as to whether other eggs would show similar effects, and the frog's egg was selected for further study. Yolk-plug and later stages, as well as early cleav-

ages, were observed in various population densities. The present inquiry is part of an extensive study concerning the effects of population density on organisms that is being conducted by Professor Allee and his associates.

MATERIALS AND METHODS

Frogs (*Rana pipiens*) were secured from Wisconsin and kept at approximately 6° C. in glazed earthenware jars, lined with moist paper. They were used within 5 or 6 weeks after being received. Frogs with eggs unovulated could not be secured from outside sources after the first of May, but females put at 6° about

¹ The writer wishes to acknowledge her gratefulness to Professor W. C. Allee for his interest in and advice on this problem.

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the first of May still produced eggs showing a high percentage of fertility on June 1. At the time of injection frogs were placed in a constant-temperature room that was held at approximately 16°. The methods of Rugh (1934), with modifications, were used in obtaining and fertilizing eggs. Fresh, finely ground-up frog pituitary was suspended in water at a concentration of one female or two male pituitaries to 1 cc. of water. During the winter each female received two subcutaneous injections of two pituitaries each, 24 hours apart. In the spring one pituitary at each injection was sufficient to induce ovulation. From 36 to 48 hours after the first injection the eggs could be obtained by stripping the female. Even though fertilization would not take place or the fertility was low the first day that they were available, the unstripped eggs often showed a high percentage of fertility 2-5 days later and for a period of 1 week thereafter. The physiology of the eggs did not change so long as the fertility remained high—at least relative to the characters studied in this paper. Females in which eggs had been ovulated were kept at 6° until about 15-30 minutes before the eggs were stripped, when they were placed at 16°. Only data from experiments in which the cleavage was 65 per cent or over are reported in this paper, except for some experiments described in Table 2, where the percentage of fertility is recorded. In many cases the fertility was very nearly 100 per cent.

A sperm suspension was prepared by macerating the testes from uninjected male frogs in approximately 8 cc. of water. The suspension was freed of all pieces of testis tissue and allowed to stand at least 20 minutes at 16° before use. It was diluted or used directly, depending on the concentration. Such a suspension gave high fertility for 2 days if kept at 6°.

During the first half of the work, testes from two or three males were frequently combined in making up a sperm suspension; later, larger males were used, and one was sufficient. Often one experiment included several females, but the eggs from different ones were kept separate throughout the experiment and were suitably distributed between experimentals and controls. Eggs from only one female were used in any one paired comparison. No marked, consistent individual differences showed up in an analysis of cases where two or three females were used in the same experiments.

In most cases the eggs were stripped into a clean glass dish, usually a Petri dish. In a few cases they were stripped onto paraffin paper. The sperm suspension was poured on and left, as a rule, for 10 minutes, although it could be left for from a few seconds to 30 minutes. The eggs were drained, rinsed twice, and then allowed to stand well covered with water. The jelly swelled to such an extent that, when the eggs were crowded, they had to be loosened from the bottom. In some experiments the eggs were separated, by means of needles, into groups or single eggs and placed in other dishes. This could not be done with safety until approximately 1 hour after fertilization. Between 5 and 30 seconds after the sperm suspension is added, frog eggs become susceptible to shaking or touching and will not cleave if disturbed. At the end of 30 minutes they can be loosened from the bottom but will not stand being cut apart. Cutting can be done without preventing cleavage of some or all of the eggs only after the eggs have turned. Because of this period when the eggs cannot be manipulated, the methods for cleavage study used in this paper can be separated into two groups: those in which the eggs were bunched or scattered at the time of fertilization, before the

sensitive period; and those bunched or scattered 1 hour after fertilization, following the sensitive period. As will be seen, the results differ in the two cases. Differences in methods will be pointed out for each series of experiments when the results are presented.

Three different types of dishes were used. In many of the experiments the eggs were placed in jelly glasses that held 125 cc. of water. The diameter of the bottom was $2\frac{1}{2}$ inches, and 135 eggs could be placed in a single layer over the bottom. Glass-stoppered Erlenmeyer flasks of 100-cc. capacity were also used, as were liter flasks with rubber stoppers.

Eggs killed in the cleavage stages were killed during either the second, third, fourth, or fifth cleavages; and they were under experimental conditions 300–600 minutes. Eggs left until the yolk plug had formed and the blastopore was closing were under experimental conditions 40–60 hours. Scattered eggs were at least approximately 1 cm. apart. Bunched eggs either were not cut or were shoved together as closely as possible.

The time for killing was determined for yolk-plug stages by direct observation. For cleavages a separate dish was placed under the same conditions as the rest of the eggs and handled as little as possible. At killing time, most of the water was decanted, and the eggs were poured through a funnel into a 100 per cent formalin solution, diluting the solution to approximately 12 per cent; this solution, in turn, was diluted further, to approximately 3 per cent formalin, within an hour. The amount of 100 per cent formalin used depended on the number of eggs to be killed. Bunched eggs were usually in a layer one egg thick; and, since they presented less surface for the killing agent to penetrate than single eggs did, they might have been killed more slowly. Evidence that such a differ-

ence is not significant in the present work is presented later. Eggs used in paired comparisons were killed at the same time; or, if there was a few seconds' lapse between killing, the scattered eggs were always killed last. For instance, in some comparisons scattered eggs from four dishes were considered as one member of a paired comparison, and eggs bunched in one dish were the other member. It was possible to tip three dishes of eggs into formalin at one time, one bunched and two scattered; then, as soon as possible, but actually a few seconds later, the other two scattered lots were killed.

An effort was made to kill the cleaving eggs when 50 per cent of the control eggs had gone through the cleavage in question—either the second, third, fourth, or fifth cleavage. Results of the counts on fixed eggs are recorded in percentage cleaved for any one cleavage studied. Eggs were recorded as cleaved when the first indication of a cleavage furrow appeared. There was no selection of data near 50 per cent cleavage. Except in one or two paired comparisons, data were not used where either the experimental or the control eggs had not begun or had already completed cleavage. Under the conditions of these experiments, after cleavage started, approximately 4 per cent more eggs began cleavage each minute until all were cleaved. This estimation is based on observation of the second cleavage.

Yolk plugs were measured with an ocular micrometer which magnifies one and one-quarter times. Two diameters at right angles were obtained in micrometer units, and for comparative purposes the product was used as a relative area, without calculating the actual area. Since the eggs were killed when the product of the diameters was in some cases over 100 and in others under 10 and since the change in size per hour is much greater when the

plugs are large, the differences in size of plug between experimental and control lots of eggs were changed into time intervals, for comparative purposes. Eggs in a wide range of yolk-plug stages were killed at hourly intervals and a graph made from the results (see Fig. 1). From this graph one can determine for any size yolk plug the expected change per hour and thus approximately translate the measured difference between the bunched and scattered eggs into hours. Too few counts were made to give an accurate curve. Also, the long-range changes in temperature in the experimental room prevent these translations into time from being anything more than a rough approximation.

The entire experimental procedure after transferring the male and female frogs from the 6° room was carried out in a large constant-temperature room having a floor space of 7 × 8 feet and a ceiling 10 feet high. The temperature was maintained by circulating conditioned air. Dishes containing eggs to be used in paired comparisons were placed as close together as possible. Flasks were stoppered, jelly glasses were covered with glass squares, and a whole tableful of covered jelly glasses was again covered with a box to prevent differences in temperature due to eddies in the circulating air. As a rule, the temperature did not vary during any one experiment, judging from observations of a thermometer calibrated to 1° C., but ranged from 12° to 17° over long periods. Experimental dishes were kept covered, as far as possible, except in one set of experiments, which will be pointed out later.

Synthetic pond water was used exclusively in the experiments. It was made up in 20-liter lots by adding the following chemicals of reagent quality: 50 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg. of $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, 50 mg. of NaNO_3 , and 50 mg. of K_2SO_4

to a liter of distilled water. The distilled water was known to be of good quality from tests of conductivity and from use in other experiments in the laboratory (Allee, Finkel, and Hoskins, 1940). The prepared "pond" water was brought to equilibrium with the air at 16° by bubbling through it, for at least 12 hours, compressed air that had been washed in distilled water. A lower CO_2 content was obtained by the continuous aeration of the water during the experiment by

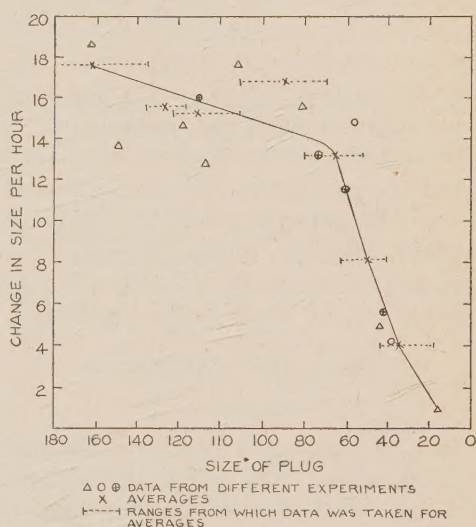


FIG. 1.—Changes, in size per hour, of yolk plugs of various sizes. Size is in micrometer units and is a relative area. See text, p. 18.

means of air passing through a train containing NaOH , followed by distilled water. The eggs were protected from the agitation resulting from the aeration process. If a higher CO_2 concentration was desired, "pond" water saturated with CO_2 at 6° was added. The CO_2 was obtained from the reaction of HCl and CaCO_3 and then washed through distilled water. The concentrations desired required from 1 to 10 cc. per liter of CO_2 -saturated "pond" water. Carbon dioxide tensions were measured by the Seyler (1894) method, using a 0.01 N

solution of Na_2CO_3 in titrating. The CO_2 values so obtained may be a little low, according to Papp (1943).

The data were analyzed statistically by the paired comparison method of "Student" (1925). Each comparison consisted of one experimental and one control set of eggs. In the accompanying tables the average of these comparisons per experiment is recorded, as well as the average of all comparisons, regardless of the number of experiments from which they were obtained. The P -values are calculated on the basis of comparisons, not as averages of experiments. The term P (probability) is an expression of the number of times one could expect as great a difference as observed by chance alone. If P equals 0.05, there are 5 chances in 100 of obtaining as great a difference by random sampling. When there are 5 or less chances in 100, the difference is considered to be statistically significant.

OBSERVATIONS AND RESULTS

CLEAVAGE

a) *Eggs bunched or scattered at the time of fertilization.*—The following methods were used in the first set of experiments, designed to determine whether grouped eggs cleave more rapidly than do isolated ones. The same methods were repeated another year to confirm the results. Ten to several hundred eggs were stripped into a Petri dish; the sperm suspension was poured on; and the dish was shaken so that some eggs were scattered and some were left in small or larger bunches one layer thick over the bottom. After 20 minutes the sperm suspension was removed; the eggs were washed and water was poured on; then the eggs were loosened from the bottom. About 1 hour after fertilization the eggs turned, and the scattered and the grouped eggs were removed and arranged in separate jelly glasses. An important point to note

here is that the eggs which at the time of fertilization became separated from the others, or those at the edges of the bunches, were selected and used as isolated eggs, while the bunched eggs were usually obtained by cutting up larger groups into smaller ones. Thus the eggs were really bunched or scattered from the time of fertilization but were placed in separate dishes an hour afterward.

It can be seen from Table 1 that under these conditions there is a significant tendency for bunched eggs to cleave more rapidly. The data from work done in two different years are tabulated on different lines. The comparisons for the first year are divided on the basis of size of group. This is of interest in the light of later experiments. The larger and smaller groups of eggs are not significantly ahead of the scattered eggs, although a trend is indicated. Since in these experiments the isolated eggs were manipulated more than the bunched ones, the bunched eggs in 2 of the 1943 experiments were partially cut apart; that is, they were not cut enough so that they became separated. This did not prevent the bunched eggs from cleaving sooner. Other experiments on this question of the effect of cutting will be discussed later. A total of 93 comparisons from 12 experiments listed in Table 1 show that, on the average, the bunched eggs were ahead of the scattered ones by 8.2 per cent cleavage. This difference has a P -value of 0.0000414. In these experiments, as in the next set, the bunched, uncut eggs did not present so much surface to the killing agent and might not have been killed so quickly as single eggs. To test this possibility, one experiment was carried out in which the bunched eggs were cut before killing, and these were also ahead of the single eggs.

Seventeen more experiments were per-

formed in which the eggs were isolated or bunched from the time of fertilization. The results are given in Table 2. The eggs, some isolated and some grouped, were left in the dish in which they were fertilized—not removed to other dishes with isolated and bunched eggs separated. Two methods were used in getting the eggs scattered or bunched. Results from the two methods were alike and have been combined in Table 2. Either the eggs were shoved around just prior to or immediately after fertilization, before they became sensitive to disturb-

although a tendency in that direction was present. Small bunches of 2–6 eggs showed a significantly faster cleavage for high plus low fertility comparisons but not for high fertility comparisons alone or for comparisons based on only 1 or 2 scattered eggs. These observations, together with those in Table 1, indicate an optimal-sized bunch of 7–20 for an increase in rate of cleavage. The larger bunches were consistently not significantly ahead. The smaller bunches were not so consistent, but results indicate that eggs in these small bunches

TABLE 1

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN EGGS BUNCHED AND EGGS SCATTERED AT THE TIME OF FERTILIZATION AND THEN PLACED IN SEPARATE DISHES 1 HOUR AFTER FERTILIZATION

DATE	NO. IN BUNCH	NO. OF EXPTS.	No. of EGGS USED IN COMPARISONS		No. OF COMPARISONS	No. of COMPARISONS IN WHICH BUNCHED minus SCATTERED IS IN PERCENTAGE CLEAVED			BUNCHED minus SCATTERED IN PERCENTAGE CLEAVED		P-VALUE FOR COMPARISONS
			Scattered	Bunched		Over +0.6	+0.5 to -0.5	Over -0.6	Av. of Expts.	Av. of Comparisons	
1943.....	4-6	6	694	1,673	14	9	2	3	+ 1.2	+ 5.6	0.2516
	7-20				43	28	4	11	+ 7.0	+ 7.1	0.0164
	21-63				22	11	2	9	+ 8.6	+ 5.6	0.1246
1944.....	6-19	6	174	268	14	10	0	4	+18.1	+16.3	0.0058

ance, to get them into the desired scattered or grouped arrangements, or the eggs were only shaken and never touched. Comparisons in which the fertility of the eggs is below 65 per cent are treated separately. Also, comparisons based on only 1 or 2 scattered eggs are not included in the first analysis but are placed in separate lines; they include eggs with both high and low fertility. As in the first set of experiments, there was a tendency for bunched eggs to cleave sooner. The difference is statistically significant in all cases for bunches that range in size between 7 and 21 eggs. Bunches larger in size did not in any case cleave significantly faster than scattered eggs,

cleave less rapidly than do those in bunches of the indicated optimal size.

If all the comparisons between eggs bunched or scattered at the time of fertilization and not touched thereafter (all those in Table 2) are combined, we find 146 comparisons; with the bunched eggs 10.6 per cent ahead; the *P*-value for the difference is 0.0000962.

Throughout the experiments reported in this paper, frequently the eggs isolated at the time of fertilization had a low fertility, compared to the grouped ones in the same dish. Fertility in this connection means that the eggs did not cleave; however, they may have been fertilized. The fertility of the bunched

TABLE 2

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN EGGS BUNCHED AND EGGS SCATTERED AT THE TIME OF FERTILIZATION AND LEFT IN THE SAME DISH TOGETHER

DATE	No. OF EXPTS.	No. OF EGGS USED IN COM- PARISONS		No. OF COM- PARISONS	No. OF COM- PARISONS IN WHICH BUNCHED <i>minus</i> SCATTERED IS IN PERCENTAGE CLEAVED			BUNCHED <i>minus</i> SCATTERED IN PERCENTAGE CLEAVED						P-VALUE OF COM- PARISONS	
								Av. of Expts.			Av. of Comparisons				
		Scat- tered	Bunched		Over +0.6	+0.5 to -0.5	Over -0.6	Size of Bunch							
								2-6	7-21	22-63	2-6	7-21	22-63		
1943 and 1944	Fertility above 65 per Cent														
	9	129	135	21	13	0	8	+ 7.6			+11.0			0.1046	
	15	340	839	44	28	2	14		+14.0			+10.5		0.0278	
	7	132	381	14	7	0	7			+ 4.4			+ 5.0	0.3844	
1943 and 1944	Fertility of Bunched Eggs, above 65 per Cent; That of Scattered Eggs, below 65 per Cent														
	3	38	38	7	5	0	2	+14.0			+16.0			0.0908	
	4	56	193	11	8	1	2		+21.0			+20.0		0.0158	
	5	51	244	7	5	0	2			+10.6			+15.0	0.2754	
1943 and 1944	Combination of Above Data for Eggs with High and Low Fertility														
	10	167	173	28	18	0	10	+ 8.6			+12.3			0.0300	
	19	396	1,031	55	36	3	16		+16.0			+12.6		0.0027	
	12	183	625	21	12	0	9			+ 7.0			+ 8.6	0.1252	
1943 and 1944	Comparisons Based on One or Two Scattered Eggs, Regardless of Fertility														
	10	25	68	17	7	4	6				- 0.3			0.6	
	8	23	224	16	9	1	6					+17.0		0.0298	
	9	12	272	9	5	0	3						+8.1	0.5652	

TABLE 3

DIFFERENCE IN PERCENTAGE FERTILE BETWEEN THE BUNCHED AND SCATTERED EGGS FOR THE EXPERIMENTS SUMMARIZED IN TABLE 2

FROM EACH EXPERIMENT EXTREMES IN FERTILITY OF EGGS FOR COMPARISONS ARE SELECTED AND AVERAGED				LIMIT OF PERCENTAGE FER- TILE BELOW WHICH NO COMPARISONS USED	
Highest Percentage Fertile		Lowest Percentage Fertile			
Bunched	Scattered	Bunched	Scattered	Bunched	Scattered
98	90	86	76	65	65
93	55	81	40	65	5

and scattered eggs has been analyzed for the experiments shown in Table 2, in an effort to determine whether the fertility is related to the rate of cleavage. See Table 3, where two points can be noted: first, that the scattered eggs were always lower in fertility than the bunched eggs, a condition which may or may not have been the result of the greater mechanical disturbance of single eggs, compared with that of bunched ones, at the time of fertilization; and, second, that when the fertility of bunched eggs dropped 5 per cent between the so-

t -value of 1.7 and P equals 0.08913. Although this later P approaches significance, it is based on only 5 cases at the lower fertility. The question needs further investigation.

b) Eggs not bunched and scattered until 1 hour after fertilization.—In the next series of experiments eggs were fertilized in a Petri dish and were kept, as far as possible, in one large group one layer thick. After they turned, about 1 hour after fertilization, the eggs were cut apart, and those which had not turned were removed. The eggs were mixed up

TABLE 4

DIFFERENCE IN RATE OF CLEAVAGE BETWEEN EGGS THAT WERE CUT APART 1 HOUR AFTER FERTILIZATION AND THEN SHOWN INTO BUNCHES AND THOSE CUT APART AND THEN ISOLATED

NO. OF EGGS PER DISH		NO. OF EXPTS.	NO. OF EGGS USED IN COMPARISONS		NO. OF COM- PARI- SONS	NO. OF COMPARISONS IN WHICH BUNCHED minus SCATTERED IS IN PERCENTAGE CLEAVED			BUNCHED minus SCATTERED IN PER- CENTAGE CLEAVED		P-VALUE FOR COMPARI- SONS
Grouped	Scat- tered		Grouped	Scat- tered		Over +0.6	+0.5 to -0.5	Over -0.6	Average of Expts.	Average of Compari- sons	
5	5	7	460	460	82	46	5	31	+2.1	+3.0	0.2713
10	5	10	910	910	91	39	1	51	-1.7	-1.8	0.3681
20	5	13	1,560	1,560	79	48	3	28	+1.4	+3.2	0.0574
40	5	9	1,020	960	48	23	2	23	+0.4	0.0	
10	5	9	260	390	26	11	0	15	-4.8	-5.0	0.1016
40	5	11	1,080	405	27	9	1	17	-4.6	-5.2	0.0456

called "high" and the "low" fertility experiments the fertility of the scattered eggs was lowered by 35 per cent. The data in Table 2 indicate that there may be a greater difference in cleavage rate between the bunched and scattered eggs in the lower-fertility comparisons. This suggests that lower fertility is associated with slower cleavage, in which case the greater difference in fertility of the bunched and scattered eggs should correlate with a greater difference in time cleavage. For comparable sets of eggs a difference of 1 between the amount of acceleration at high and at low fertility is not significant; t equals 0.1. For another set of eggs a difference of 16 has a

and counted at random into jelly glasses. In some dishes they were scattered, and in others they were pushed together in a group. The results are given in Table 4. Eggs in bunches of 5 cleaved slightly sooner, on the average, than did accompanying lots of 5 isolated ones. Despite a large number of comparisons, the difference is not statistically significant. The conditions for this particular set of comparisons differ from those for all the others, since the dishes were not covered and since they contained only 25 cc. of water instead of 125 cc. In the remaining experiments summarized in Table 4 a group of 20 eggs gave a slightly earlier appearance of the first few sets of cleav-

age furrows when compared with a similar number of scattered eggs. The observed difference approaches statistical significance. A group of 10 was slowed insignificantly, while a group of 40 was significantly retarded. This fits roughly the results obtained with eggs that were isolated or bunched from the time of fertilization, except that here cleavage in the smallest and largest bunches tested was actually retarded, as compared with the scattered eggs. In the instances summarized in Tables 1 and 2 the bunched eggs cleaved more rapidly than did the scattered eggs but seemed relatively re-

half and the two halves placed in separate jelly glasses. One of the halves was cut and the eggs scattered, and one of the halves was bunched. Half of the bunched eggs were left without cutting, and half were cut apart and then shoved together. The cut and uncut bunches both show an insignificant deviation in rate of cleavage from the scattered eggs, thus indicating that cutting had no effect on the rate of cleavage. One would expect that the bunched eggs might have been ahead of the scattered ones, on the basis of the work reported in Table 4. Perhaps the reason they are

TABLE 5

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN BUNCHED EGGS WHICH HAVE NEVER BEEN CUT APART AND SCATTERED EGGS AND THAT BETWEEN BUNCHED EGGS WHICH HAVE BEEN CUT APART AND THEN SHOVED TOGETHER AND SCATTERED EGGS

No. of Eggs per Bunch	No. of Expts.	Treat-ment of Bunched Eggs	No. of Eggs Used		No. of Compari-sons	No. of Comparisons in Which Bunched minus Scattered Is in Percentage Cleaved			Bunched minus Scattered in Per-centage Cleaved		P-Value for Compari-sons
			Scat-tered	Bunched		Over -0.6	+0.5 to -0.5	Over -0.6	Av. of Expts.	Av. of Compari-sons	
6-23.....	8	Cut	289	343	30	15	1	14	+0.5	+2.0	0.1204
15-32.....	9	Uncut	660	766	46	22	0	24	-0.6	+0.6	

tarded, as compared with egg populations that were intermediate in size. Thus, in all these cases the optimal population was intermediate in size during early cleavage.

Since in the experiments described in Table 1 the bunched eggs in most cases were not cut apart while in those described in Table 4 they were cut and then shoved together, it was thought desirable to find out if cutting eggs apart 1 hour after fertilization had any effect on the rate of cleavage. Experiments were set up in which the eggs were treated as in the experiments described in Table 4 except that all eggs were not mixed up; rather, one large bunch was cut from the rest, and then it was cut in

not is because there is too much deviation from the indicated optimal-sized bunch of 20 eggs.

A small set of experiments was carried out in which the rate of cleavage of eggs in dense populations was compared with that in sparse populations. One hundred and thirty-five eggs covered the bottom of a jelly glass, and in some cases the eggs were in two or three layers. The data are summarized in Table 6. None of the differences have statistical significance. The observed differences are small; so in order to determine whether they are significant many comparisons are needed, and at this point too few are available to come to any final conclusion.

The experiments involving eggs iso-

lated or bunched an hour after fertilization were all performed in 1944. Nine of the experiments in which eggs were bunched or isolated from fertilization and the eggs not touched (see Table 2) were also done in 1944. Nevertheless, it was thought desirable to repeat the 1943 methods (the results of which are reported in the first three lines of Table 1), in which the eggs were scattered and bunched from fertilization and removed to separate dishes 1 hour later, in order to make sure that differences between eggs isolated at the time of fertilization and those isolated 1 hour later did not

which frog eggs had developed to blastula or to tail-bud stages. The conditioning eggs were kept in loosely covered dishes with as little water as possible, in an effort to keep the CO_2 concentration from rising; and since they were in a single layer, all developed at about the same rate. The supernatant liquid was milky. Eggs placed in this water were slower to cleave than in untreated water, provided the conditioned water was used immediately after the conditioning eggs were removed. The treated water produced no effect on cleavage if it was allowed to stand exposed to the air for a

TABLE 6
RATE OF CLEAVAGE OF EGGS IN DENSE AND SPARSE POPULATIONS

NO. PER DISH		NO. OF EXPTS.	NO. OF EGGS USED		NO. OF COMPARISONS	NO. OF COMPARISONS IN WHICH BUNCHED <i>minus</i> SCATTERED IS IN PERCENTAGE CLEAVED			RATE IN DENSE <i>minus</i> RATE IN SPARSE (AV. OF COMPARISONS)
Sparse	Dense		Sparse	Dense		Over +0.6	+0.5 to -0.5	Over -0.6	
4-27	52-97	8	310	897	12	4	1	7	- 0.4
7-42	107-133	8	437	731	6	3	0	3	+ 1.2
4-37	170-187	4	241	713	4	2	0	2	- 3.0
8-20	247-286	2	137	533	2	0	0	2	-12.0

arise from some unrecognized change in technique of treating the eggs between the two years. When the 1943 methods were repeated, the results were the same as before. They are reported in the last line of Table 1. Bunches of 5-19 were found to start cleavage significantly ahead of scattered eggs. This indicates that differences in results when eggs were bunched and scattered at different times are related to the time at which the eggs were placed under experimental conditions and not to changes in treatment of eggs between the two years.

Preliminary experiments were carried out in which the rate of cleavage was measured in the usual, uncontaminated pond water, as compared with the rate in conditioned water, that is, water in

day or two before being tested. The retarding agent is either unstable or volatile. Carbon dioxide is known to retard cleavage (Merwin and Allee, 1943) and may have been the causative agent here in spite of efforts to keep its concentration at a minimum.

YOLK PLUG

In experiments set up to determine whether the blastopore would close more rapidly in bunched eggs than scattered ones, the following methods were used. In most cases the eggs were cut apart 1 hour after fertilization and equal numbers placed in like volumes of water. In half of the dishes the eggs were scattered, and in half they were bunched. In some cases, contributing 13 compari-

sons to the 80 included in Table 7, both the scattered and the bunched eggs were attached to a piece of paraffin paper and the paper placed in a flask. In these cases the bunched and scattered eggs were in the same flask. The eggs were allowed to develop to the yolk-plug stage with no change of water. Measurement of the plugs showed that the bunched eggs had developed faster, on the average, than had scattered eggs (see Table 7). Size differences were transposed into time differences by the use of the graph shown in Figure 1. Two of the experiments included in Table 7 contained quite a

indicated that the range of decreasing stimulation by CO_2 comes at around 3.9 cc. per liter of CO_2 . The values given for the size of the yolk plugs that developed in water aerated by air approximately freed from CO_2 , as compared with those in control flasks aerated by untreated laboratory air, are not based on micrometer measurements (Table 8, line 1). The eggs were killed when the blastopore was closing, at which time they were easily graded into stages by eye and given arbitrary values. These gradings were checked and rechecked, and the results fit into a trend. The same grading

TABLE 7

RATE OF CLOSURE OF BLASTOPORE OF BUNCHED EGGS COMPARED WITH THAT OF SCATTERED EGGS WHEN THERE WAS AN EQUAL NUMBER OF EGGS PER DISH

No. OF EGGS PER FLASK*		No. OF EXPTS.	No. OF EGGS USED IN COMPARISONS		No. OF COMPARI- SONS	CLOSURE OF BUNCHED <i>minus</i> CLOSURE OF FEWER (HOURS' DIF- FERENCE IN DEVELOP- MENT)		P-VALUE FOR COMPARI- SONS
Scattered	Bunched		Scattered	Bunched		Av. of Expts.	Av. of Comparisons	
4-44	4-56	6	608	918	80	+0.2	+0.3	0.0051

* 100-cc. or 1,000-cc. flasks; all containers were alike for any given experiment.

number of comparisons. In one of these experiments the bunched eggs were slower in development, but not significantly so. The other of these experiments, as well as the total of 6 experiments when considered together, showed a significantly more rapid closure of the blastopore for the bunched eggs.

A part, if not all, of the indicated increased rate of development of the bunched eggs may have been caused by a heightened CO_2 concentration around the grouped eggs, for CO_2 speeds the rate of yolk-plug closure (see Table 8). A preliminary experiment had indicated that a CO_2 tension as high as 7.3 cc. per liter was stimulating; however, 2 experiments with many comparisons

method was used on a series of eggs killed at regular intervals, so that size differences could be transposed into the time differences, as shown in Table 8, line 1.

If, in stoppered flasks or covered dishes with an equal amount of water, the number of eggs was increased, there would be more CO_2 with more eggs, and the eggs would be expected to develop faster in the yolk-plug stage. This was found to be the case. In one series of 7 flasks the number of eggs was increased from 8 to 84, and the CO_2 was measured after the eggs were taken out (see Table 9). It can be seen that, with 39 or more eggs per flask and with the CO_2 1.9 cc. per liter or over, the plugs were definite-

ly smaller. The increase in the amount of CO_2 in the flasks was not in proportion to the increased number of eggs present. The reason for this is not clear; it may be related to the alkaline reserve of the eggs, or it might be a result of bacterial action. Twenty-one comparisons in 7 experiments, including those in the experiment reported in Table 9, are presented in Table 10. In these com-

parisons the number of eggs per dish is larger for one member of the pair. The eggs are significantly faster in development at the yolk-plug stage in the flasks containing more eggs. This tabulation includes both bunched and isolated eggs, but bunched are always compared with bunched and isolated with isolated.

In the data presented in Table 11 the scattered or bunched condition and

TABLE 8
EFFECT OF CO_2 ON THE RATE OF CLOSURE OF THE BLASTOPORE

NO. OF EXPTS.	TYPE OF DISH	NO. OF FLASKS	NO. OF EGGS	NO. OF COMPARISONS	P-VALUE FOR COMPARISONS	CLOSURE AT HIGH CO_2 CONCENTRATION minus CLOSURE AT LOW CONCENTRATION (HOURS' DIFFERENCE IN DEVELOPMENT)			
						CO_2 High*			
						0.7	3.0	3.9	6.5
						CO_2 Low			
						0.0	0.7	0.7	0.7
I.....	Liter flask	14	979	9	0.0108	+0.4†			
I.....	Liter flask	18	331	54	0.00014		+0.4		
I.....	100-cc flask	28	250	32	0.00137		+0.9		
				32	0.1096			+1.0	
I.....	100-cc. flask	27	263	59	0.00031			+0.4	
				64	0.1096				+0.2

* Concentrations of CO_2 in cubic centimeters per liter.

† Eggs not measured with micrometer.

TABLE 9
INCREASE IN CO_2 CONCENTRATION AND SPEED-
ING-UP OF YOLK-PLUG CLOSURE AS THE NUM-
BER OF EGGS DEVELOPING PER CLOSED 100-CC.
FLASK IS INCREASED

No. of Eggs per Flask	CO_2 in Cubic Centimeters per Liter	Area* of Plugs
0.....	0.7	
8.....	1.1	44
9.....	1.1	45
14.....	1.2	50
28.....	1.7	44
39.....	1.9	38
66.....	2.0	41
84.....	2.7	34

* Area is in micrometer units and is the product of the two diameters (see p. 18).

the number of eggs per dish vary, always with the bunched eggs present in larger numbers per dish. Here both local and general increasing of the CO_2 should take place, and so the stimulation should be greater than with either condition alone. There was stimulation, but one cannot analyze the relative role of the bunching and the increase in number of eggs per volume by comparisons with former observations made on the effect of these two factors separately (Tables 7 and 10), because of the variability in numbers of eggs present per dish and the differences in the volumes of water in which the eggs were contained.

In all the comparisons between yolk plugs so far, the bunched eggs were in rafts one layer thick or had an interrupted top layer. If the eggs were completely cut off from the surface, as they were in the center of a mass of eggs, the central ones were retarded in development at the yolk-plug stage, as compared with those at the surface. For ex-

CO₂ was higher with 77 eggs per flask than when only 12 were present. Three more cases of eggs surrounded by others are listed in Table 12.

Other rafts or bunches of eggs have been observed in order to check on these findings. When a raft of eggs two or three layers thick was left barely covered with water, the eggs in the second layer from

TABLE 10
RATE OF CLOSURE OF BLASTOPORE IN RELATION TO NUMBERS PRESENT PER GIVEN VOLUME

No. OF EGGS PER DISH*		No. OF EXPTS.	No. OF EGGS USED IN COMPARISONS		No. OF COMPARI- SONS	MORE PRESENT <i>minus</i> FEWER (HOURS' DIF- FERENCE IN DE- VELOPMENT)		P-VALUE FOR COM- PARISONS
Fewer	More		Fewer	More		Av. of Expts.	Av. of Comparisons	
1-39	5-84	7	790	962	21	+1.2	+1.7	0.0000†

* 100-cc. flasks, 1,000-cc. flasks, or jelly glasses holding 125 cc.
† 1 is 11.

TABLE 11
RATE OF CLOSURE OF BLASTOPORE OF BUNCHED EGGS WITH MORE EGGS PER FLASK COMPARED
WITH THAT OF SCATTERED EGGS WITH FEWER PER FLASK

No. OF EGGS PER DISH*		No. OF EXPTS.	No. OF EGGS USED IN COMPARISONS		No. OF COM- PARISONS	BUNCHED, MORE <i>minus</i> SCATTERED, FEWER (HOURS' DIFFERENCE IN DEVELOPMENT)		P-VALUE
Scattered, Fewer	Bunched, More		Scattered, Fewer	Bunched, More		Av. of Expts.	Av. of Comparisons	
2-7	15-22	4	198	314	40	+1.1	+0.6	0.00032

* Jelly glasses hold 125 cc. of water.

ample, in one bunch of 77 eggs, 33 were on the surface of the bunch and 27 were more or less inclosed; and 14 on the bottom were probably completely surrounded. The outer eggs were 0.6 hour ahead of the inner eggs in development and approximately 12 hours ahead of the ones on the bottom. The outer eggs were also 0.2 hour ahead of eggs in a comparable flask with only 12 eggs per flask. This would be expected, since the

the top were slower in developing, and the bottom ones were still slower. If eggs were observed in the neural-fold stage, a center-edge difference showed up even in a raft one layer thick. The neural folds of the center eggs were slower to close.

HATCHING

Hatching is the next stage at which a clear-cut end-point can be found. According to Cooper (1936), hatching is

under the control of a secretion. Observations on hatching in one series of flasks showed that in a bunch of 50 eggs several hatched earlier than did any eggs in groups of 5 and that in the groups of 5 some eggs hatched about 12 hours before the isolated eggs began to hatch. Despite the considerable difference in the time of hatching, no difference in the developmental stage reached by the larvae from single eggs or from bunches of 5 could be distinguished when hatched larvae from single and grouped eggs were killed at the same time and observed under a binocular microscope.

One set of flasks was set up containing scattered eggs in various densities. The eggs were allowed to develop to hatching; then all were killed and examined and the CO_2 content of the water determined. In a flask in which the CO_2 concentration reached 9.0 cc. per liter, the larvae died at the time of hatching, perhaps owing to lack of oxygen. When there were fewer per flask and the CO_2 reached 6.9 cc. per liter, the larvae lived. Their gills were very slightly less developed than those in a still sparser population, where the final CO_2 concentration measured 1.4 cc. per liter. Thus CO_2 , in the long run, would not seem to effect development greatly—at least under the conditions tested where the water was not changed.

DISCUSSION

The present work on frog eggs was suggested by the observations of Allee and Evans (1937*a* and *b*) on *Arbacia* eggs. They found an optimal density for rate of cleavage and early development.

CLEAVAGE

When frog eggs were under experimental conditions from the time of fertilization, the bunched eggs were found to cleave more rapidly than scattered

ones. An optimal-sized bunch is indicated in which the stimulation is greater than for either larger or smaller bunches. If eggs were not grouped or isolated until 1 hour after fertilization, there was an indication of stimulation for bunches of 20, but larger or smaller groups generally showed retardation in rate of cleavage relative to scattered eggs. The difference in the amount of time the eggs were exposed to experimental conditions in these two cases would not seem to be the cause of the different results; rather, there must be some special environmental condition or reactivity of the egg

TABLE 12

RETARDATION OF DEVELOPMENT OF FROG EGGS
IN THE YOLK-PLUG STAGE WHEN THEY ARE
SURROUNDED BY OTHER EGGS IN A MASS

No. of Eggs			OUTER minus INNER (HOURS' DIFFERENCE IN DEVELOPMENT)
Total	Inner	Outer	
132	29	103	+5.9
131	37	94	+4.8
53	9	44	+0.7

existing during the first hour after fertilization.

Peebles (1929), working on *Arbacia* eggs, observed that water in which eggs had developed depressed cleavage. Allee and Evans (1937*b*) also observed this, and work reported in the present paper suggests that the same is true for frog eggs. Peebles noted speeding of cleavage when eggs were exposed to a dialyzate from cleaving eggs. She also found extracts of *Arbacia* eggs that hastened cleavage. Allee *et al.* (1942) obtained an extract of cleaving *Arbacia* eggs that stimulated cleavage. Finkel, Allee, and Garner (1942) have discussed the possibility that copper, given off by the eggs after fertilization, may be the effective agent. No work has been done on *Arba-*

cia, in which the factor or factors causing acceleration at optimal density is shown to be limited in action to a short time following fertilization; this might hold if copper was the effective agent.

Needham (1942) reports that Ashbel and, later, others have observed that an acid is produced soon after the fertilization of echinoderm eggs. One would expect this increase in acid to depress groups, since it would release CO_2 from the carbonates in the surrounding water and since CO_2 is known to depress the rate of cleavage of *Arbacia* eggs (Smith and Clowes, 1924; Haywood and Root, 1930).

Certain differences between frog and *Arbacia* eggs need to be kept in mind, even though, at present, they are hard to evaluate in relation to rate of development. The sea-urchin egg has already gone through both maturation divisions, while the frog egg has yet to give off the second polar body after the sperm enters. The fertilization membrane rises in *Arbacia* in 4 minutes, while in the frog egg it takes three-quarters of an hour to an hour at 16°C . under experimental conditions.

When frog eggs are bunched or scattered at fertilization, a number of factors, not present in the work on *Arbacia* or in the work on frog eggs that are scattered or bunched an hour after fertilization, are introduced, differentiating between the bunched and scattered eggs. These factors must be considered as possible reasons for the differences observed between eggs isolated or bunched at fertilization and those similarly treated an hour later. Scattered eggs show a lower fertility, a state that may be related to the fact that these eggs may be more shaken about than the bunched eggs at the time the dish is shaken to get the eggs bunched and scattered. It has been pointed out earlier that eggs are very

sensitive to mechanical disturbance after fertilization. Disturbance prevents cleavage and may slow it, but this point has not been settled in the present work. There might be a differential rate of penetration of the bunched and scattered eggs by the sperm; the scattered eggs are more thoroughly washed; also, they are faster to turn.

Another possible difference between isolated and bunched eggs during this period deserves brief attention. According to Needham (1931), Bialaszewicz and Bledowski report that freshly ovulated frog eggs give off a considerable amount of CO_2 . Needham (1942) also reports that Dalcq and Pastells and Brachet find that much of the CO_2 is discharged in the oviduct and that, only when the CO_2 is low enough, will fertilization take place. The CO_2 might become lower sooner in the isolated eggs and fertilization take place sooner; then they should cleave sooner. The CO_2 given off by the grouped eggs would raise the CO_2 tension around them and slow the appearance of the first furrow, since any increase in CO_2 has this effect (Merwin and Allee, 1943). In both these cases the expected results would be a slowing of the bunched eggs relative to the scattered eggs, and this is contrary to the evidence reported in this paper. Any increase in CO_2 has been shown to delay the appearance of the first few cleavage furrows for *Arbacia* eggs (Smith and Clowes, 1924, and Haywood and Root, 1930); it has the same effect on the cleavage of frog's eggs. In spite of this, denser populations of *Arbacia* eggs and groups of frog eggs cleave more rapidly than sparse populations or scattered eggs.

Osmotic conditions around scattered eggs may differ from those around bunched ones during the first hour. Needham (1931) reports the work of several investigators that indicates that

after fertilization frog eggs give off salts into the perivitelline space. Although no reports have been found in the literature regarding the diffusion of substances out into the surrounding water from the perivitelline space during the first hour after fertilization, McClendon (1915) showed that salts diffuse out at a much faster rate during the first 7 hours after fertilization than later. Voss (1926) found that the perivitelline space of embryos in the medullary fold stage (the youngest stage he observed) were smaller in crowded eggs than in less crowded ones.

Allee *et al.* (1942) do not think that the heat of metabolism is an important factor in accelerating the rate of cleavage in denser populations of *Arbacia* eggs, and no proof that it is important in causing bunches of frog eggs to cleave more rapidly than scattered ones could be obtained in the present experiments. The observed differences in rates of cleavage for frog eggs, isolated or bunched at fertilization, would require approximately 0.06° C. difference in temperature, based on the effect of temperature on the development of *R. pipiens* as determined by Atlas (1935). Observations with two Beckman thermometers, calibrated to each other and placed under various conditions, indicated that a range of 0.03° C. between dishes might exist, but not differentially for experimental and control dishes. When one thermometer was placed among eggs or was coated with eggs, it did not reveal any consistent change in temperature when compared with the other thermometer.

YOLK PLUG

By the time the yolk-plug stage is reached, the reaction to CO_2 has changed; and, while increased CO_2 tension depresses cleavage, it accelerates blastopore closure. It has other processes to work on

besides cleavage—for instance, migration of cells. Approximately 3.0 cc. per liter of CO_2 caused most rapid development at this stage. Later, in the adult body, the cells operate at a much higher CO_2 concentration.

Bellamy (1922) observed stimulation of the development of *R. pipiens* in the yolk plug and also in the larval stages in a N/5,000 and N/20,000 HCl solution in well water. He suggested that CO_2 released from the carbonates by the acid was the stimulating substance. In his experiments the water was changed every day, while this was not done in the present experiments. This may explain the fact that Bellamy obtained stimulation lasting to hatching, while this stimulation did not continue in the present experiments to any measurable extent. Vernon (1895), working with echinoderm larvae, observed a slight trend toward stimulation by CO_2 ; and he found that concentrations up to the critical one did not interfere with development to any extent.

HATCHING

It has been pointed out that bunched eggs hatch sooner than isolated ones. Cooper (1936) obtained a hatching secretion by removing the membranes from eggs and allowing the eggs to develop to the stage at which they would normally hatch. The enzyme obtained in this manner may be concentrated enough to dissolve the jelly from other eggs in 1 hour. Bliss (1940) found that 10 per cent trypsin would produce the same effect and did not affect the rate of development but that development stopped in the presence of tryptic digest products. Shumway (1940) observed that embryos in his Stage 19 hatched if they were shaken, while those in his Stage 20 hatched spontaneously. In the present work, groups of 5 eggs hatched sooner than isolated ones, and observa-

tions of the larvae killed after all had hatched revealed no difference in development. If groups were larger, the center ones were retarded in development; and these did hatch sooner, relative to their growth stage—at least for Shumway's Stage 19 or earlier; but accurate staging was not carried out. The earlier hatching, relative to the development of the larvae, may be due to the earlier stimulation of the larvae to move and rupture the membranes or to a more effective action of the enzyme, owing to a higher concentration, caused by crowding, or to more favorable chemical conditions around crowded eggs.

The present study extends our knowledge about optimal populations to the early development of another species—this time a vertebrate. The wider aspects of these and related phenomena are discussed by Allee (1938). The rate of development is a favorable indicator for use in such population studies and may well have ecological significance, as will be discussed later. Observed modifications to date are relatively small in most cases and consist of slight variations in rate of cleavage or in rate of later development. These are often, but not always, temporary and subject to later compensation.

The egg cell is not dependent on other cells for growth-stimulating substances, the need of which in the case of body cells shows up in tissue culture. Bacterial cells are often dependent on each other in that a certain size inoculum is necessary for survival and growth. Garrod (1936) suggests, in explanation, that certain bacterial cells produce a substance necessary to each other and must have it in a certain concentration. Eggs shed into the water are independent of other organisms except sperm of the same species. *Arbacia* eggs are known to secrete substances relative to fertilization,

such as fertilizin (Lillie, 1919); but these are probably not significant in the present study and can be excluded with certainty for *Arbacia* eggs and for frog eggs isolated an hour after cleavage. Other substances known to be produced during the early development of the *Arbacia* eggs are copper and an acid; these have been discussed. Other metabolic products, such as CO_2 , ammonia, and urea (Brachet, 1939), or special secretions, such as hatching enzymes, may become concentrated around grouped eggs and modify the rate of development or the time of hatching.

If group-initiated modifications were steadily advantageous generation after generation, one would expect selection to have resulted in eggs being laid in masses of some size. The fact that some species of animals—frogs among them—lay their eggs in bunches may be an indication of such advantages. The benefit derived from bunching that is most frequently mentioned in the literature is that the large mass of jelly holds heat absorbed from the sun's rays by the darkly pigmented embryos and so keeps the temperature of the mass above that of the surrounding water. Moore (1939) notes that clumps of eggs are characteristic of early spring breeders—at least in the temperate zone. The significance of this is not clear, although a possible temperature effect may be indicated.

The indication from the work reported in this paper of a sensitive period following fertilization suggests that bunched eggs might be more protected from mechanical injury during that time. This possible mechanical protection of eggs in bunches suggests certain comparisons with the advantages of a large graft in transplanting tissues when mechanical or sudden chemical changes may harm small grafts more than large ones (Waterman, 1936). Developmental

acceleration of grouped eggs by CO_2 , such as has been reported in the present paper for the yolk-plug stage, would not seem to be important under ordinary circumstances. Significant speeding of total development or of hatching could be of importance to frogs when the eggs are laid in ponds which dry up or in which the water level may fall.

Other types of biotic relations, including predation, may also have affected the evolution of the laying of eggs in groups. The survival values involved are complex and call for experimental analysis rather than a priori speculation.

Whatever the larger implications, certain definite results have been obtained as a result of the present study. These will be summarized in the following paragraphs.

SUMMARY AND CONCLUSIONS

Frog eggs (*R. pipiens*) that were bunched at the time of fertilization cleaved more rapidly than those that were scattered. Bunches ranging in size from 7 to 20 eggs were consistently significantly ahead of scattered eggs; those of from 21 to 60 eggs in size were not significantly ahead, although a trend may have been present. There are indications that bunches smaller than 7 eggs were less accelerated than those of 7-20 eggs. Thus, an optimal-sized bunch accelerates the first few cleavages. The percentage of fertility of the scattered

eggs was lower than that of the bunched eggs.

If the eggs were not placed in groups or scattered until an hour after fertilization (after the eggs turned), there was a slight, almost significant, tendency for the groups of 20 eggs to cleave faster; but both smaller and larger groups cleaved slower than accompanying scattered eggs; the groups of 40 eggs, significantly so. The depressing effect of bunching under these conditions may be the result of an increased CO_2 concentration. The factor or factors causing acceleration are not known.

Closure of the blastopore took place sooner in eggs in a group one layer thick than in scattered eggs, and it was faster if there were more eggs per volume of water. This speeding of development was probably the result of an increased CO_2 concentration. A content of approximately 3 cc. of CO_2 per liter was more effective than higher or lower concentrations in speeding the closure of the blastopore.

If eggs were in the center of a mass, they were retarded in the yolk-plug stages and later. Such eggs have not been studied in the cleavage stages. The central eggs in a group one layer thick were noticeably retarded in the neural-fold stage and later.

Grouped eggs hatched sooner than isolated ones, provided the groups were not so large that development was retarded for the centrally placed embryos.

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